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Generation of Cerebellar Interneurons from Dividing Progenitors in White Matter

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Summary

The traditional view of the external granular layer of the cerebellar cortex giving rise to interneurons has been challenged by recent studies with quail-chick chimeras. To clarify the time and site of origins of interneurons, a retrovirus carrying the β-galactosidase gene was injected into the deep cerebellar tissue or external granular layer of postnatal day 4/5 rats to label dividing progenitors. After deep cerebellar tissue injection, unipolar cells were found initially in white matter at 2 days postinjection and subsequently in the internal granule and molecular layers 4–6 days postinjection. Morphologically defined basket, stellate, and Golgi neurons were clearly identified by 20 days postinjection. In contrast, retroviral labeling of cells in the external granular layer produced only granule neurons in the internal granule layer. Thus, dividing progenitors in the cerebellar white matter migrate through the white matter into the cortex before differentiating into a variety of cortical interneurons.

Introduction

The developing cerebellum contains two distinct proliferative zones from which cerebellar cortical neurons are derived. Progenitors of Purkinje cells arise in the primary germinal zone, the ependymal layer in the roof of the fourth ventricle, and migrate radially through the deep cerebellar structures late in gestation (Uzman, 1960; Miale and Sidman, 1961; Altman and Bayer, 1978). Granule cells arise from the second germinal zone, the external granule layer (EGL), which lies just beneath the pial surface, covering the cerebellar folia. Granule cell progenitors migrate inward through the molecular layer (ML) into the internal granule cell layer (IGL) during early postnatal life (Jacobson, 1991; Ramon y Cajal, 1995). In contrast to these well-described pathways for Purkinje and granule cell development, the genesis of the interneurons of the cerebellar cortex has been controversial. Golgi interneurons originate from the same area as the Purkinje cells and migrate into the IGL in late gestation (Uzman, 1960; Miale and Sidman, 1961). However, the neuroepithelium at the roof of the fourth ventricle is no longer in a proliferative state after birth, whereas the EGL still is (Miale and Sidman, 1961). Thus, the basket and stellate interneurons generated during the first two postnatal weeks have been traditionally thought to be derived from progenitors in the EGL (Altman, 1972; Jacobson, 1991). Recent studies using quail-chick chimeras and transplantation of early postnatal EGL progenitors have countered this view, concluding that EGL cells give rise exclusively to granule cells and, therefore, that basket and stellate interneurons are derived from the primary germinal zone at the base of the cerebellum (Hallonet et al., 1990; Hallonet and Le Douarin, 1992; Gao and Hatten, 1994).

It has been unclear, however, how to reconcile this view with the cessation of proliferative activity in the primary germinal zone by the time interneurons develop. Although such proliferative activity has disappeared by birth, many dividing cells reside in the cerebellar white matter (WM) of young rodents (Uzman, 1960; Miale and Sidman, 1961; Fujita et al., 1966). These proliferating cells have been thought to represent immature glia (Uzman, 1960; Miale and Sidman, 1961; Fujita et al., 1966) and are likely to have been generated from the subependymal layer at the roof of the fourth ventricle, but to have maintained their proliferative capabilities as they migrate through the cerebellum (Miale and Sidman, 1961; Fujita et al., 1966). Whether this population of dividing cells also contains neuronal progenitors has not been determined.

The introduction of replication-deficient recombinant retroviruses that carry marker genes, such as β-galactosidase (BAG), has provided a new approach to study cell fate and development. Retroviral genes are incorporated into the genomic DNA of dividing cells and remain in one of the daughter cells and its progeny (Sanes et al., 1986; Price, 1987; Hajihosseini et al., 1993). Thus, retroviral labeling provides two major advantages over ^{3}H-thymidine labeling: first, the morphology of the labeled cells can be readily observed, due to the expression of a marker gene; and second, the inserted retroviral genes become heritable markers, so that one can trace the fates of infected cells.

To clarify the origin and to define the sequence of events in the development of the interneurons in the cerebellar cortex, we have used a BAG retrovirus to label dividing cells in the deep cerebellar tissue or the EGL of postnatal day 4/5 (P4/5) rat pups. The distribution pattern and the morphological changes of the labeled cells at 2, 5, and 20 days postinjection (dpi) were examined and compared. We demonstrate that the interneurons in the cerebellar cortex are not derived from the EGL as traditionally assumed, but come from dividing progenitors in the early postnatal cerebellar WM. These progenitors migrate through the WM into the cortex before they show any morphological signs of differentiation. Cells of EGL produce only granule neurons in the IGL.

Results

Proliferating Cells in the Cerebellar WM Give Rise to Cortical Interneurons

We initially injected BAG retrovirus stereotactically into deep cerebellar WM of P4/5 rat (Figure 1), according to

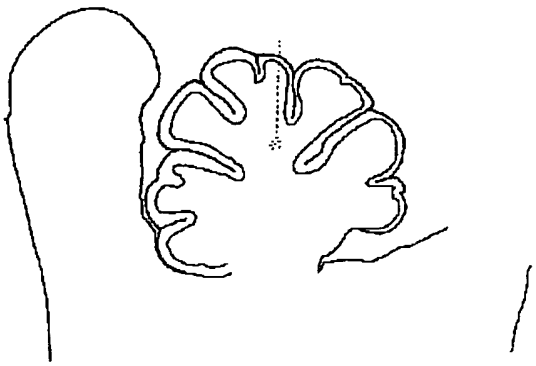

Figure 1. Camera Lucida Drawing Showing the Injection Site in the Deep Cerebellum

This is a drawing of an aldehyde-fixed parasagittal section obtained from a P5 rat sacrificed immediately after a stereotactic injection of 0.2 microliter of trypan blue, using the same coordinates as those employed for BAG injection. The grouped spots represent several light-blue-stained cells in the WM around the injection site. The dotted line represents the path of the micropipette.

the coordinates noted in Experimental Procedures. The folia of the cerebellum have not yet fully developed at this time (Heinsen, 1977), which allowed us to inject into the WM easily. Injection sites were consistently found between the folia and the deep cerebellar nuclei. Proliferating cells 2 dpi were revealed by X-Gal staining and appeared as simple unipolar cells that resided mostly in WM (>90% per section) in several folia (Figure 2). A few cells with a similar immature morphology were also found in the IGL, the Purkinje cell layer (PCL), and the ML. No labeled cells were found in the EGL or in the subependymal layer located at the roof of the fourth ventricle. No obvious cerebellar tissue damage due to the BAG injection was observed (see comments in Experimental Procedures).

From 4 to 6 dpi, more labeled cells had appeared in the ML, PCL, and IGL (Figures 3 and 4A), although the

Figure 2. Deep Cerebellar Injection of BAG-Labeled Progenitors Mostly in WM at P6, 2 dpi

X-Gal-stained unipolar cells that resided mostly in the WM in several folia. A few cells were in the IGL, PCL, and ML. No EGL cells were labeled. Magnification, 38×. Inset: BAG-labeled cells in the WM were small and elongated and each displayed a single short process. Magnification, 240×.

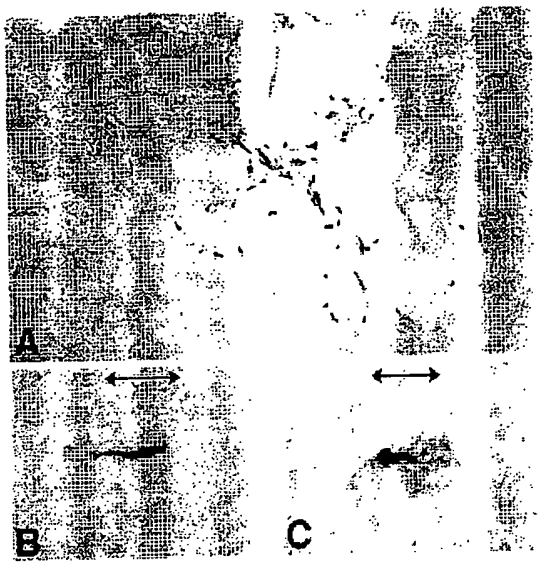

Figure 3. BAG-Labeled Immature Cells in ML, PCL, and IGL at P10, 5 dpi

(A) Many unipolar cells have appeared in the ML, PCL, and IGL. The majority of the labeled cells still resided in the WM and remained immature. Magnification, 29×.

(B) One of the many unipolar cells in ML oriented parallel to the pial surface.

(C) One of the few more complex cells in ML. The orientation of the pial surface is marked by arrows in (B) and (C). Magnification, 243×.

majority of the labeled cells still resided in WM and appeared immature. Similarly, many of the labeled cells in the ML were unipolar and either radially or horizontally oriented (Figure 3B). However, some cells were bipolar or bore several thin processes and fine branches (Figures 3C and 4A), suggesting initial differentiation during this period of time. No labeled EGL cells or granule cells were found.

The labeled cells in the ML became more complex by 8–10 dpi (Figure 4B). A few unipolar cells were still observed during this stage. The labeled cells were found in both upper and lower portion of the ML in several folia. Many cells had medium-sized cell bodies and processes, which extended in several directions (Figure 4B). A few of the cells were situated near the PCL and displayed both processes that projected to the pial surface and other lateral processes that projected along the PCL (Figure 4B). These cells probably represent immature basket interneurons, since they show essential morphological features of well-defined basket cells at 20 dpi (see below). Although many cells in the ML had a neuronal morphology (Figure 4B), it was difficult to identify the types of these cells based on their morphologies.

By 20 dpi, clearly recognizable neuronal forms had emerged in the ML (Figures 4C–4E; Figure 5), and the unipolar cells had disappeared. Many of the cells with perikarya in the lower aspect of the ML displayed the morphological features of basket interneurons described previ usly (Altman, 1972; Rakic, 1972; Ramon y Cajal, 1995). They had cell bodies of polygonal, triangular, or stellate shape and were situated near the PCL

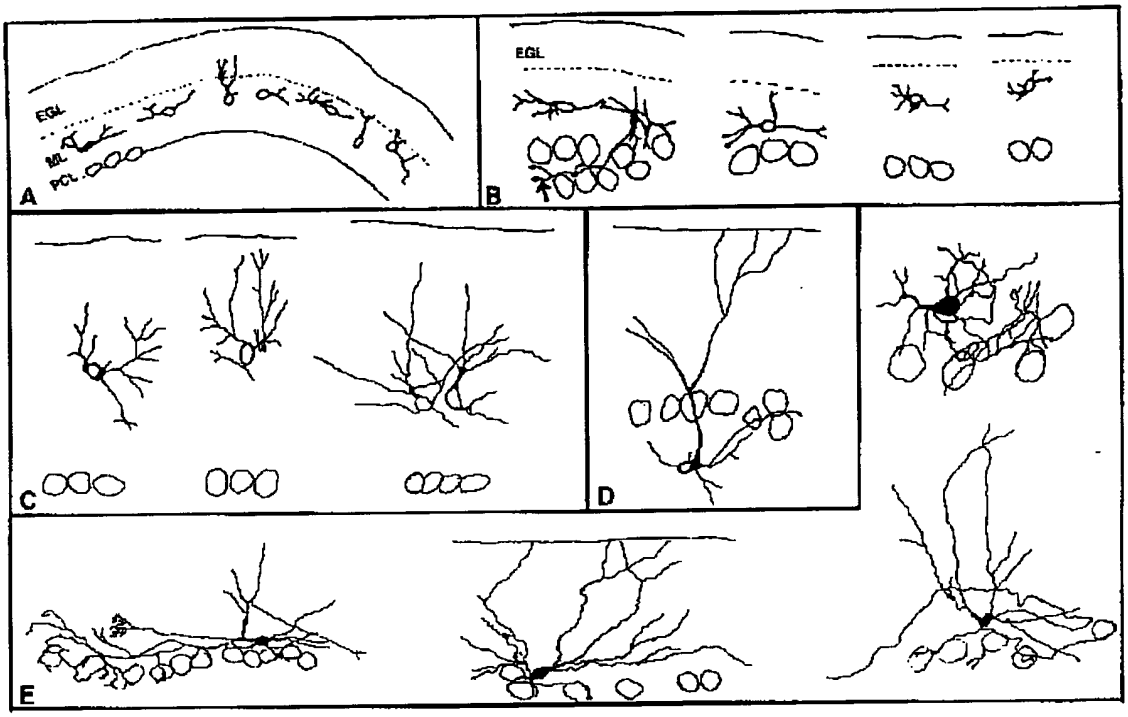

Figure 4. Camera Lucida Drawings of BAG-Labeled Cells in the ML at Different Developmental Intervals after Retroviral Injection at P5

(A) Unipolar and multiprocess cells at 6 dpi. (B) More complex multiprocess cells at 8–10 dpi. Note that a lateral process arising from the relative immature basket cell body (arrow) projected along the Purkinje cells (drawn as ovals). (C) Stellate cells at 20 dpi. (D) Golgi interneuron at 20 dpi. (E) Basket cells at 20 dpi.

(Figures 4E, 5B, and 5D). Their processes extended in all directions, but mainly toward the pial surface. A thin lateral process that emerged from the parikaryon sent secondary or tertiary branches into the PCL and formed "baskets" around Purkinje cells (Figures 4E, 5B, and 5D). Some cells in the middle or upper aspects of the ML scattered irregularly and displayed the morphological features of stellate cells, with relatively small cell bodies and fewer shorter processes projecting both toward the pia and toward the PCL (Figures 4C and 5C). Occasionally, cells with a Golgi appearance were observed in the IGL. These cells displayed relatively large perikarya and several processes oriented in different directions. The processes penetrated through the PCL into the ML and to the pial surface (Figures 4D and 5E). Purkinje cells, as well as neurons in deep cerebellar nuclei, which are generated and migrate embryonically (Altman and Bayer, 1978; Jacobson, 1991), were never labeled. Labeled granule neurons were also not found.

When BAG injections were performed at P14, labeled cells were initially observed with simple immature morphology in the WM at 2 dpi. No basket, stellate, or other types of interneurons were detected in the ML by 17 dpi, however (data not shown).

Four types of cells in the cerebellar cortex contain th inhibitory transmitter γ-aminobutyric acid (GABA) and its synthetic enzyme glutamate decarboxylase (GAD): Purkinje, basket, stellate, and Golgi cells (Hokfelt and Ljungdahl, 1972; McLaughlin et al., 1974; Saito et al., 1974; Kelly et al., 1975; McGeer et al., 1975; Wood et al., 1976; Oertel et al., 1981). To confirm that the labeled cells in the ML were interneurons, we performed double immunofluorescence staining with antibodies against β-gal and GAD, the enzyme that specifically expressed in GABAergic neurons (Patel et al., 1985). Approximately half of the β-gal-positive cells in the ML were GAD positive (Figure 6). They bore several processes and resided in upper, middle, and lower portions of the ML.

Besides these interneurons in the ML and IGL, a variety of glia including oligodendrocytes, Bergmann glia, velate astrocytes of the IGL, and astrocytes in WM were also clearly identified by their morphologies in their appropriate layers (see Figure 5A). Thus, glial cells are also generated from dividing progenitors in the deep cerebellar WM. A more detailed study on the migration and maturation of glia will be reported separately (L. Zhang and J. E. Goldman, unpublished data).

EGL Cells Give Rise Only to Granule Neurons

To determine directly whether cells of the EGL also give rise to interneurons, we labeled dividing EGL cells at P5 by a superficial injection of BAG virus. By 2 dpi, labeled cells of the EGL (Figure 7A, thin arrow), spindle-shaped cells with radially oriented processes (characteristic of migrating granule cells) in the EGL and ML (Figure 7A, thick arrow), and cells within th IGL were all found (Figure 7A). By 6 dpi, labeled cells were found in the IGL (Figure 7B). The labeled cells had small (5–8 μm), round

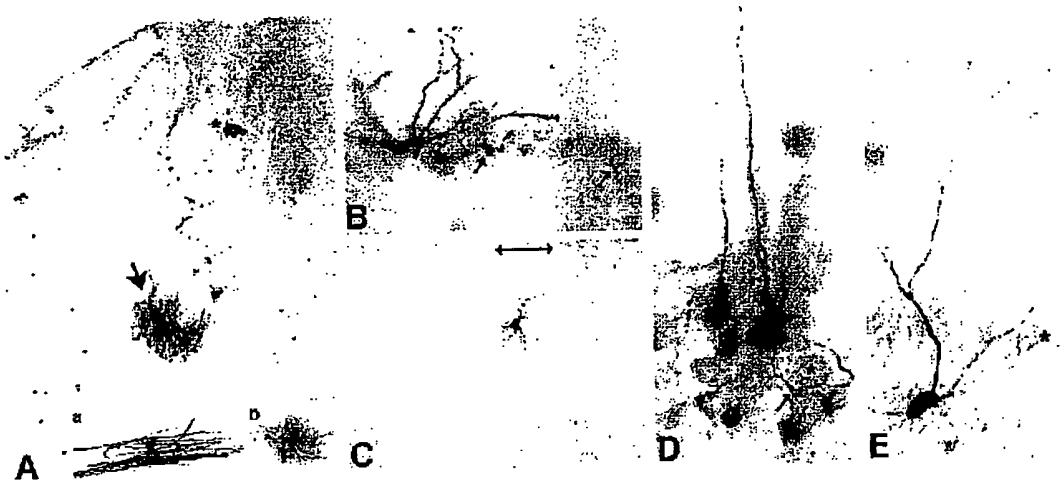

Figure 5. P25 BAG-Labeled Cells in the ML and IGL, 20 dpi

(A) Most of the cells appeared differentiated: interneurons, Bergmann glia (asterisk) oligodendrocytes (inset a and arrow), and astrocytes (inset b). No unipolar cells were found. (B and D) Two basket cells in the ML, forming "basket" (small arrows) around Purkinje cells (asterisks). (C) A stellate cell in the ML. (E) A Golgi neuron in the IGL. Magnifications, 27× (A); 41× (insets a and b); 95× (B and C); 244× (D and C).

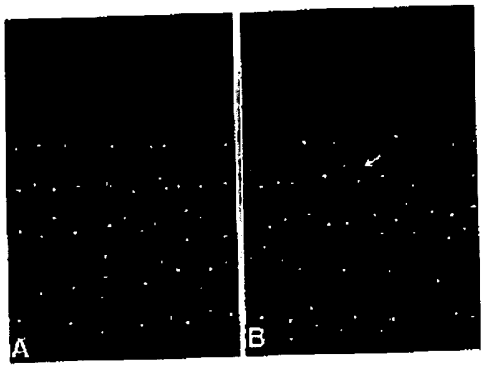

Figure 6. Double Immunofluorescence Stained P25, 20 dpi, with β-gal and G4D Antibodies

(A) β-gal$^+$ cell in the ML. (B) The same cell was GAD$^-$ (small arrow). Magnification, 205×.

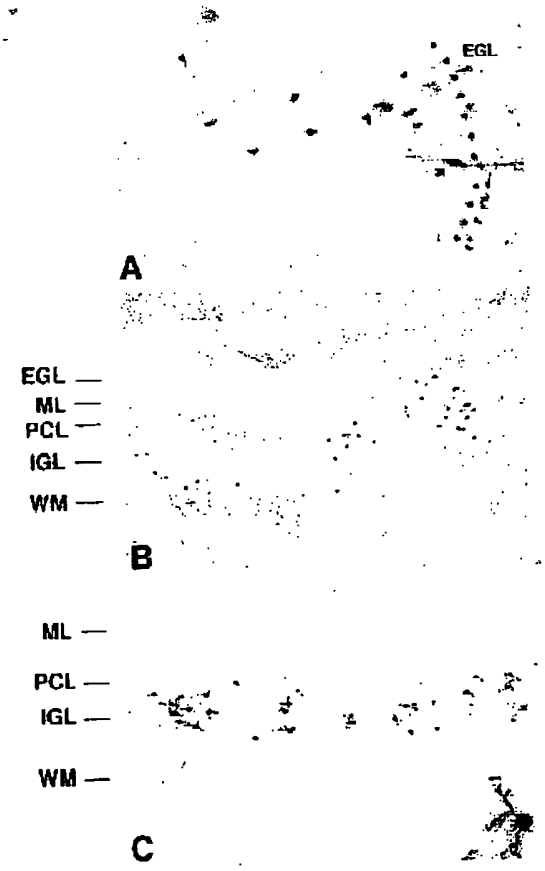

Figure 7. BAG Injection into the EGL Labeled at P5 Labeled Only Granule Cells

(A) BAG-labeled EGL cells (thin arrow) were seen in the EGL (curved dotted line) at 2 dpi. Migrating cell (thick arrow) and cells within the IGL were also seen. Magnification, 106×. (B) 6 dpi and (C) 20 dpi BAG-labeled cells appeared in the IGL and displayed the morphology of small, round granule cells, including the characteristic "claw" feature. (Inset). No labeled cells were detected in the ML or WM. Magnifications, 55× (B and C); 219× (inset).

cell bodies. The processes with the claw-shaped terminal ramification, typical of granule cells, were seen among some of the labeled cells. By 20 dpi, labeled cells were found only in the IGL (Figure 7C). Most of them displayed the characteristic claw-shaped terminal ramifications of mature granule cells (Figure 7C, inset), as described previously (Eccles et al., 1967; Ramon y Cajal, 1995). No basket and stellate cells or other interneurons were observed in the cerebellar cortex after EGL injections.

Discussion

Interneurons Arise from Dividing Progenitors in the Deep Cerebellum

The most significant finding of this study is that the int neurons of the cerebellar cortex, such as basket,

stellate, and Golgi cells, are derived from dividing neuronal progenitors in th postnatal cerebellar WM. Immature cells of the EGL produced only granule cells in the IGL and did not produce any interneurons. It has been difficult to distinguish clearly the origins of the various cell types (both neurons and glia), owing to the dual germinal zones in the cerebellum. The BAG virus infected a small number of dividing cells in a restricted area in a relatively short period of time. These features allowed us to label dividing progenitors in WM and in the EGL separately and to follow their migratory patterns and fates, thus providing an approach to identify clearly the origins of different cell types in the cerebellum.

The classic view that interneurons in the ML arise from the EGL has been challenged recently by quail-chick chimera studies (Hallonet et al., 1990; Hallonet and Le Douarin, 1992) and EGL cell transplantation (Gao and Hatten, 1994). Supportive findings have also been obtained from a different group also using quail-chick chimeras (Otero et al., 1993) and a recent study that shows the presence of basket and stellate cells in the *meal mea* mutant mouse, which has a significantly reduced EGL and, therefore, a paucity of granule cells in the IGL (Napieralski and Eisenman, 1993). However, none of the above studies shed light on the time of origin, site of origin, and migratory paths of these interneurons. By using thymidine dating experiments in the chick, Hanaway (1968) suggested that all the interneurons of the ML are derived from the primary neuroepithelium, but such a conclusion has not been drawn for the mammalian cerebellum, because the basket and stellate cells formed at a time when the proliferative activity in the primary germinal zone has already ceased (Miale and Sidman, 1961; Altman 1972). Our study thus extends earlier findings by demonstrating that postnatally generated interneurons are not derived from the primary germinal zone directly, but rather from dividing neuronal progenitors in the early postnatal cerebellar WM. Assuming that these cells arise from the ventricular zone, they must migrate into deep WM and continue to divide there until they eventually reach the ML (see below).

^{3}H-thymidine labeling studies have described immature, dividing cells in the deep WM in the postnatal cerebellum (Uzman, 1960; Miale and Sidman, 1961; Fujita et al., 1966). The dividing cells in WM have been thought to represent immature glia, rather than neurons, for two reasons. First, glial cells can continue to divide after they migrate out of the germinal zone, and some of them retain such capability throughout life (Allen, 1912; Smart and Leblond, 1961; Altman, 1972). Second, it has been a general rule that neuronal progenitors become postmitotic before they migrate out of germinal zones (His, 1904; Sauer, 1935; Sauer and Walker, 1959; Jacobson, 1991). In this study, we have confirmed that cerebellar glial cells indeed originate from progenitors in cerebellar WM. However, our observations also demonstrate the existence of dividing neuronal progenitors that eventually give rise to interneurons of cerebellar cortex in the same area. Migration of dividing neuronal progenitors is an exception t the general rule, although the genesis of neurons in nongerminal zones can also be found in the olfactory bulb and hippocampal dentate gyrus of postnatal rodents (Altman and Das, 1965; Angevine, 1965; Altman, 1966; Hinds, 1968; Hinds and Hinds, 1968; Luskin, 1993; Lois and Alvarez-Buylla, 1994).

A variety of glia are also derived from the progenitors in the early postnatal rat cerebellar WM (see Figure 5A). The population of immature cells we have labeled with BAG therefore consists of cells that will eventually differentiate into interneurons, oligodendrocytes, and the various types of astrocytes. We do not yet know whether this progenitor population is composed of multipotent cells able to give rise to both glia and interneurons or whether the neuronal progenitors are committed to a neuronal fate. Several studies suggest the existence of multipotential progenitors that can generate both neurons and glial cells (Turner et al., 1990; Williams et al., 1991; Reynolds et al., 1992; Reynolds and Weiss, 1992; Nakafuku and Nakamura, 1995). If there are separate progenitors, they are likely to divide and possibly to migrate together.

Migration and Differentiation of Interneuron Progenitors

What is the source of these dividing progenitors that appear in the deep WM, and what migratory route do the progenitors take? Considering the facts that immature progenitors are initially found mainly in the WM and subsequently in the cortex and that the EGL produced only granule cells in IGL, it seems most likely that interneuron progenitors originate in the germinal zone at the base of the cerebellum, in the roof of the fourth ventricle, and from there migrate into WM and then into cerebellar cortex. Such a migratory path has been suggested for cerebellar oligodendrocytes (LeVine and Goldman, 1988; Reynolds and Wilkin, 1988; Warrington and Pfeiffer, 1992; Gonye et al., 1994).

How long the progenitors of interneurons remain in the WM after they leave the germinal zone is unknown. We observed that labeled progenitors remained mainly in the WM from 1–3 dpi (e.g., Figure 2), and the majority of the cells were still in the WM between 4–6 dpi and remained immature (see Figure 3). It seems that a large number of the immature cells of the early postnatal rat cerebellum remain in the WM for a short period of time (at least ~2 days) before they migrate out of the WM into the IGL, PCL, and ML. It is possible that progenitors migrate into WM at an earlier time and remain there as a dividing population until an appropriate later time to colonize the cortex.

Most of the progenitors of interneurons appear as simple unipolar cells as they migrate from the WM into the cerebellar cortex (e.g., Figure 3). They retain this morphology until they arrive at the ML. There, they undergo a series of morphological transformations that culminate in the mature interneuron. The BAG-labeled cells follow the sequence of morphological changes through which interneurons pass in the ML, as described by S. Ramon y Cajal and others (summarized in Rakic, 1972): from initially simple bipolar cells oriented parallel to the pial surface, to more complex process-bearing cells, to fully developed neurons. The early unipolar forms were not initially described, perhaps because for some reason cells in such an immature stage do not acquire the Golgi impregnation or did not show specific morphological features that would allow identification.

The migratory interneuron progenitors must move through the IGL and PCL before entering the ML. We do not know whether this travel is directed by specific substrates, such as axonal pathways, for example. Once in the PCL, progenitors may ascend into the ML along Bergmann glial processes. Although granule neurons from the EGL migrate along Bergmann glial fibers into the IGL (Rakic, 1971, 1972), neurons can migrate along radial fibers bidirectionally, at least in vitro (Edmondson and Hatten, 1987; Hatten, 1990). Thus, Bergmann glia may not confer a directionality of migration, but rather serve as a permissive track; migrating cells will move in the direction in which they begin until they reach a "stop" signal. Once in the ML, interneuron progenitors encounter parallel fibers. This encounter provides a major determinant of interneuron differentiation (Rakic, 1972).

The rodent cerebellar ML is a narrow band, and it is thus difficult to draw conclusions about specific sequences of colonization and differentiation of interneurons therein. However, in the primate ML, interneuron differentiation proceeds from the PCL toward the EGL (Rakic, 1972, 1973). If we can extrapolate our results to primates, we would suggest that interneuron progenitors, arriving in the ML from below, colonize progressively more superficial layers of the ML. Neuronal progenitors must therefore migrate through layers of differentiating interneurons and bundles of parallel fibers to reach their eventual destinations. This pattern resembles that of the developing neocortex, in which progressively migrating neuroblasts colonize more superficial positions (Sidman et al., 1959; Uzman, 1960; Angevine and Sidman, 1961; Bayer and Altman, 1987).

Morphological signs of interneuron differentiation began by 4–6 dpi, and some morphological identifiable immature basket cells were observed around 8–10 dpi (see Figure 4B). Many well-defined interneurons were observed at 20 dpi (Figures 4C–E; Figure 5). Thus, most of the labeled interneuron progenitors differentiate within two to four postnatal weeks. Unlike a previous ^{3}H-thymidine study, which concluded that the Golgi neurons complete their final mitosis before birth (Uzman, 1960), we found that the generation of Golgi neurons has not been completed by P4/5. The generation of Golgi neurons is far less frequent than that of basket and stellate interneurons by this time, however. Nevertheless, there are overlaps among the generation of different interneuron types in the early postnatal rat cerebellum.

A large number of interneurons were observed 20 days after a retroviral injection at P5. In contrast, no interneurons were detected 17 days after injection at P14, which suggests that the genesis of interneurons was greatly reduced by P14. This decrease could be due to a loss of neuronal progenitors or to the lack of appropriate neurogenic signals from the cerebellar cortex to guide the migration and/or to induce the differentiation of neuronal progenitors. Our findings are consistent with a previous ^{3}H-thymidine study, which reported that the genesis of basket and stellate cells in the ML of rat reaches a peak by P6–7 and P8–11, respectively, and then decreases rapidly (Altman, 1972).

In conclusion, retroviral labeling of dividing progenitors demonstrates that mitotic neuronal progenitors exist in the early postnatal rat cerebellar WM and migrate through the postnatal rat cerebellar WM into cortex before they differentiate into a variety of cortical interneurons. Cells of the EGL produce only granule cells in the IGL.

Experimental Procedures

BAG Injection

BAG retrovirus was collected as described previously (Levison and Goldman, 1993). Sprague-Dawley rats at postnatal day 4/5 (day of birth = P0) were anesthetized on ice for 7 min prior to the surgery. After the skin over the head was cleaned with 70% ethanol, a 0.5–1 cm long V-shaped incision was made on the skin over the occipital bone. Animals were placed on a stereotactic device (David Kopf Instruments, Tujunga, CA) attached with a vertical holder for a Hamilton syringe and kept cold with ice packs. The muscles overlying the cisterna magna were cut and gently pulled aside. A 10 μl Hamilton microliter syringe (Hamilton Co., Whittier, CA) attached to a pulled-glass micropipette was filled with stock BAG retrovirus and lowered gently through an incision (2 mm lateral from midline) and through the meninges to a depth of 0.8 mm into the deep cerebellum. Over 5 min, 1 μl of stock virus (1×10^5 cfu/ml) was injected (see Figure 1). For EGL-labeling experiments, the micropipette was lowered gently through the incision to a position just above the EGL. The wound was coated with ampicillin/streptomycin and sealed with Vetbond (Henry Schein, Inc., Port Washington, NJ). The pups were revived at 35°C and, when active and showing no signs of respiratory distress, were returned to the litter. No obvious damage due to the BAG injection was observed, except for occasional microscopic hemorrhages at the injection site and rare damaged cells, which were stained with trypan blue (Life Technologies, Inc., Grand Island, NY). BAG injections into the forebrain subventricular zone have demonstrated that the virus is not incorporated into microglia or macrophages (Zerlin et al., 1995). Furthermore, there is no increased cell proliferation around a virus injection site, as determined by BrdU labeling (J. M. Gensert and J. E. Goldman, unpublished data).

X-Gal Histochemical Staining

Animals (at least 5 animals per data point) at 2, 5, and 20 dpi were anesthetized by intraperitoneal injection of Ketamine/Xylazine mix (0.25 mg/kg; Henry Schein Pharmaceutical, Port Washington, NY) and were perfused through the left cardiac ventricle with: normal saline containing 6 U/ml heparin for 5 min followed by 3% paraformaldehyde in 0.2 M phosphate-buffered saline (PBS, pH 7.3) for 15–20 min. The brains were removed and immersed in 3% paraformaldehyde in PBS at 4°C and postfixed for 2–6 hr. A short postfixation time proved critical in allowing us to view the processes of the infected cells. Longer fixations (≥12 hr) resulted in a progressive loss of X-Gal staining in cell processes, although cell bodies were still visible after 24 hr fixation, in some cases. A series of parasagittal vibratome sections (50–100 μm) were collected and washed in PBS containing 2 mM $MgCl_2$. The sections were immersed overnight at 28°C in a solution containing 1 mg/ml X-Gal (Molecular Probes, Eugene, OR), 30 mM potassium ferricyanide, 30 mM potassium ferrocyanide, and 2 mM $MgCl_2$. After being rinsed 2 × 10 min with PBS, 1 × 10 min with PBS containing 3% dimethyl sulfoxide, and 2 × 10 min with PBS, the sections were mounted onto gelatin-coated slides and air dried. They were dehydrated through an alcohol series (50%, 70%, 95%, 95%, 100%, 100%) to xylene, coverslipped using Permount, and examined under an Olympus BH-2 microscope fitted with Nomarski optics. All experiments were carried out in accordance with institutional guidelines for animal care.

Indirect Immunofluorescence

We double stained some of the 20 dpi (P25) sections with a mouse monoclonal antibody against β-galactosidase (IgG2A, 1:20 dilution; Promega, Madison, WI) and a rabbit polyclonal antiserum against GAD (obtained from Dr. Steven W. Levison). Sections were permeabilized in 0.2% Triton X-100 in PBS for 30 min and blocked for 1 hr

In 10% BSA, 10% normal goat serum in PBS before being immersed overnight at 4°C in the primary antibody solution containing 10% lamb serum, 10% fetal bovine serum. Sections were thoroughly washed the next day and incubated with goat anti-mouse IgG2A (1:50) and goat anti-rabbit IgG (1:50) secondary antibodies conjugated with fluoresceln and rhodamine (Southern Biotechnology Associates, Birmingham, AL), respectively. Sections were examined with an Olympus BH-2 microscope equipped with epifluorescence optics.

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